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A Novel Clathrin Adaptor Complex Mediates Basolateral Targeting in Polarized Epithelial Cells

Heike Fölsch,* Hiroshi Ohno,‡ Juan S. Bonifacino,† and Ira Mellman*§

*Department of Cell Biology and
Ludwig Institute for Cancer Research
Yale University School of Medicine
New Haven, Connecticut 06520-8002
†Cell Biology and Metabolism Branch
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland 20892-5430
‡Division of Molecular Membrane Biology
Cancer Research Institute
Kanazawa University
13-1 Takaramachi
Kanazawa 920-0934
Japan

Summary

Although polarized epithelial cells are well known to maintain distinct apical and basolateral plasma membrane domains, the mechanisms responsible for targeting membrane proteins to the apical or basolateral surfaces have remained elusive. We have identified a novel form of the AP-1 clathrin adaptor complex that contains as one of its subunits μ1B, an epithelial cell-specific homolog of the ubiquitously expressed μ1A. LLC-PK1 kidney epithelial cells do not express μ1B and missort many basolateral proteins to the apical surface. Stable expression of μ1B selectively restored basolateral targeting, improved the overall organization of LLC-PK1 monolayers, and had no effect on apical targeting. We conclude that basolateral sorting is mediated by an epithelial cell-specific version of the AP-1 complex containing μ1B.

Introduction

The plasma membrane of polarized epithelial cells is differentiated into apical and basolateral domains, each with distinctive sets of proteins and lipids (Rodriguez-Boulan and Powell, 1992; Drubin and Nelson, 1996; Yeaman et al., 1999). Sorting of newly synthesized plasma membrane proteins typically occurs in the *trans*-Golgi network (TGN), where apical and basolateral components are selectively packaged into specific transport vesicles. The basic logic of polarized sorting is well known and involves a hierarchical interplay between two types of sorting determinants. Basolateral transport is often dependent on the presence of a discrete cytoplasmic domain targeting signal (Casanova et al., 1991; Hunziker et al., 1991; Le Bivic et al., 1991; Matter et al., 1992). Basolateral signals often, but not always, involve critical tyrosine or dileucine residues, suggesting at least a superficial relationship to signals that specify receptor endocytosis via clathrin-coated pits (Matter and Mellman, 1994). Apical transport, on the other hand, occurs only in the absence of a functional basolateral signal and involves N- or O-linked carbohydrate moieties in a protein's ectodomain or as yet unspecified information in the membrane-anchoring domain. These features are thought to permit segregation in glycolipid rafts that are incorporated into apical transport vesicles (Keller and Simons, 1997; Simons and Ikonen, 1997). The proteins that decode either apical or basolateral signals, however, have remained unidentified.

Since basolateral targeting signals are found in a membrane protein's cytoplasmic domain, it seems likely that they will be recognized by a cytosolic protein or protein complex. Indeed, for that subset of proteins which are localized at the basolateral surface due to the presence of a cytoplasmic tail PDZ-binding domain, interaction with cytosolic PDZ proteins has been found essential to ensure polarity. However, PDZ interactions seem to be important for selective retention after arrival at the basolateral surface as opposed to sorting into transport vesicles at the level of the TGN (Cohen et al., 1998).

In the case of the more common tyrosine-dependent basolateral sorting signals, a role for clathrin adaptor complexes has seemed a possibility, although no direct evidence has yet emerged. It is well established that tyrosine-based and dileucine-based sorting signals involved in endocytosis and lysosomal targeting are recognized by one or more heterotetrameric adaptor complexes AP-1 (γ, β1, μ1, σ1), AP-2 (α, β2, μ2, σ2), and AP-3 (δ, β3, μ3, σ3) (Hirst and Robinson, 1998; Bonifacino and Dell'Angelica, 1999). A fourth related complex, AP-4 (ϵ, β4, μ4, σ4), has recently been described (Dell'Angelica et al., 1999a; Hirst et al., 1999). AP-2 mediates endocytosis from the plasma membrane, while AP-1, AP-3, and AP-4 are thought to mediate sorting at the TGN and/or endosomal membranes. Interaction between the adaptor complexes and tyrosine-based sorting signals is mediated by the μ subunits (Ohno et al., 1995, 1996), while dileucine signals may interact with the β subunits (Rapoport et al., 1998).

We have recently identified a closely related (79% identity at the amino acid level) isoform of the μ1 subunit of AP-1 whose expression is limited to cells of epithelial origin (Ohno et al., 1999). mRNA for the new protein, designated μ1B to distinguish it from the ubiquitously expressed μ1A, was found to be expressed in various polarized epithelial cell lines, including MDCK, Caco-2, HT-29, Hec-1-A, and RL-95-2 cells. An exception was the kidney epithelial cell line LLC-PK1, thought to be derived from the renal proximal tubule. LLC-PK1 cells polarize but do not express detectable amounts of μ1B mRNA. Interestingly, LLC-PK1 cells have recently been reported to exhibit a potential "defect" in cell surface polarity (Roush et al., 1998), but the molecular nature of the defect was not defined. We have taken advantage of the functional μ1B knockout provided by LLC-PK1

§To whom correspondence should be addressed (e-mail: ira.mellman@yale.edu).

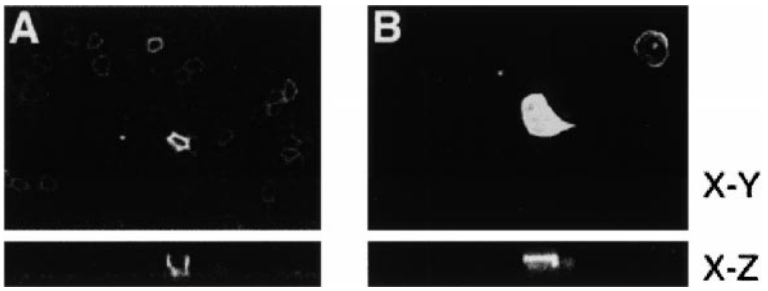

Figure 1. Sorting of LDL Receptor in MDCK Cells and LLC-PK1 Cells

MDCK cells (A) or LLC-PK1 cells (B) were grown on Transwell filters and infected with recombinant adenoviruses for 1 hr and cultured for 2 days to allow for the expression of LDL receptor. Viable cells were incubated with antibodies against the LDL receptor, fixed, and incubated with FITC-labeled secondary antibodies as described in Experimental Procedures. Specimens were analyzed by confocal microscopy. Representative X-Y and X-Z sections are shown.

cells to demonstrate that an epithelial cell-specific μ1B-containing AP-1 complex plays a critical role in basolateral targeting.

Results

LDL Receptor Is Basolateral in MDCK Cells and Apical in LLC-PK1 Cells

To determine whether μ1B plays a role in polarized sorting, we first compared the localization of the LDL receptor in μ1B-positive MDCK cells and μ1B-negative LLC-PK1 cells. The LDL receptor is well known to localize to the basolateral plasma membrane domain of MDCK cells by virtue of signals contained within its cytoplasmic tail (Matter et al., 1992, 1994). Expression was achieved by infecting polarized cells grown on filters with a recombinant adenovirus encoding the receptor (adLDLR).

Two days after infection, the cells were immunolabeled, fixed, and analyzed by confocal microscopy. In the MDCK cells, LDL receptor was detected only at the basolateral surfaces, independently of expression levels (Figure 1A). This was already apparent in X-Y confocal sections (upper panel), where the marginal outlines of expressing cells were stained. Basolateral localization was confirmed in vertical (X-Z) optical sections, where expressing cells displayed LDL receptor at the lateral and basal, but not the apical, plasma membrane domains (Figure 1A, lower panel). These control experiments with MDCK cells demonstrated that virus infection did not interfere with polarized expression of the LDL receptor. An entirely different picture was obtained for infected LLC-PK1 cells, however (Figure 1B). In these cells, LDL receptor was found only at the apical surface regardless of expression level. This is the same phenotype obtained in MDCK cells when both basolateral targeting signals are deleted from the LDL receptor cytoplasmic tail (Matter et al., 1992). These observations thus suggested a possible involvement for μ1B in basolateral targeting mediated by cytoplasmic tail signals.

Generation of μ1B-Expressing LLC-PK1 Cells

We then asked whether expression of μ1B in LLC-PK1 cells would permit the basolateral targeting of LDL receptor. For this purpose, LLC-PK1 cells were first stably transfected with an expression plasmid containing a full-length μ1B cDNA, or a μ1A cDNA as a control. To follow μ1B expression at the protein level, two μ1B-specific anti-peptide antibodies were produced in rabbits against either N-terminal or C-terminal peptides, which were only ~50% identical to the corresponding peptides in μ1A (Figure 2A). To confirm the specificity of these antibodies, they were tested by Western blot analysis against recombinant μ1A and μ1B protein produced in *E. coli*. As shown in Figure 2B, an antibody previously generated against μ1A reacted well with both μ1A and μ1B. The new anti-peptide antibodies, however, were specific for μ1B. The C-terminal peptide antibody was subsequently used for detection of μ1B expression in LLC-PK1 transfectants.

Nontransfected LLC-PK1 cells and cloned LLC-PK1

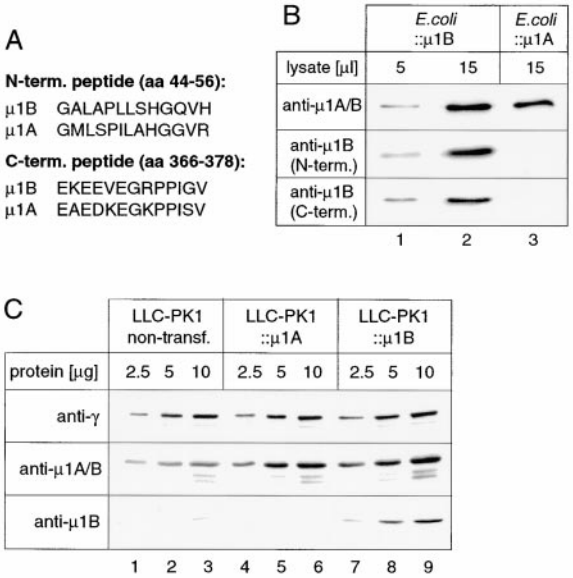

Figure 2. Analysis of LLC-PK1 Cells Transfected with μ1A or μ1B cDNAs

(A) Peptides directed against the N-terminal part or C-terminal part of the human μ1B protein (423 aa) and the corresponding regions in mouse μ1A (423 aa), which were used for immunization of rabbits for the production of μ1B-specific antibodies.

(B) Recombinant μ1A and μ1B were produced in *E. coli*, partially purified, and subjected to SDS–PAGE followed by a transfer onto nitrocellulose. Immunodecoration with an antibody directed against μ1A but cross-reacting with μ1B (upper panel) and the two μ1B-specific antibodies (lower panels) were performed to characterize the specificity of the antibodies.

(C) Nontransfected LLC-PK1 cells or LLC-PK1 cells transfected with μ1A or μ1B cDNA were lysed in RIPA buffer, and 2.5, 5, or 10 μg of total protein was used for SDS–PAGE and Western blotting (see Experimental Procedures for details). γ-adaptin, μ1A/B, and μ1B were detected by immunodecoration with anti-γ-adaptin antibodies (clone 100/3, Sigma), a cross-reacting μ1A/B antibody as in (B), and the μ1B-specific antibody raised against the C terminus of μ1B.

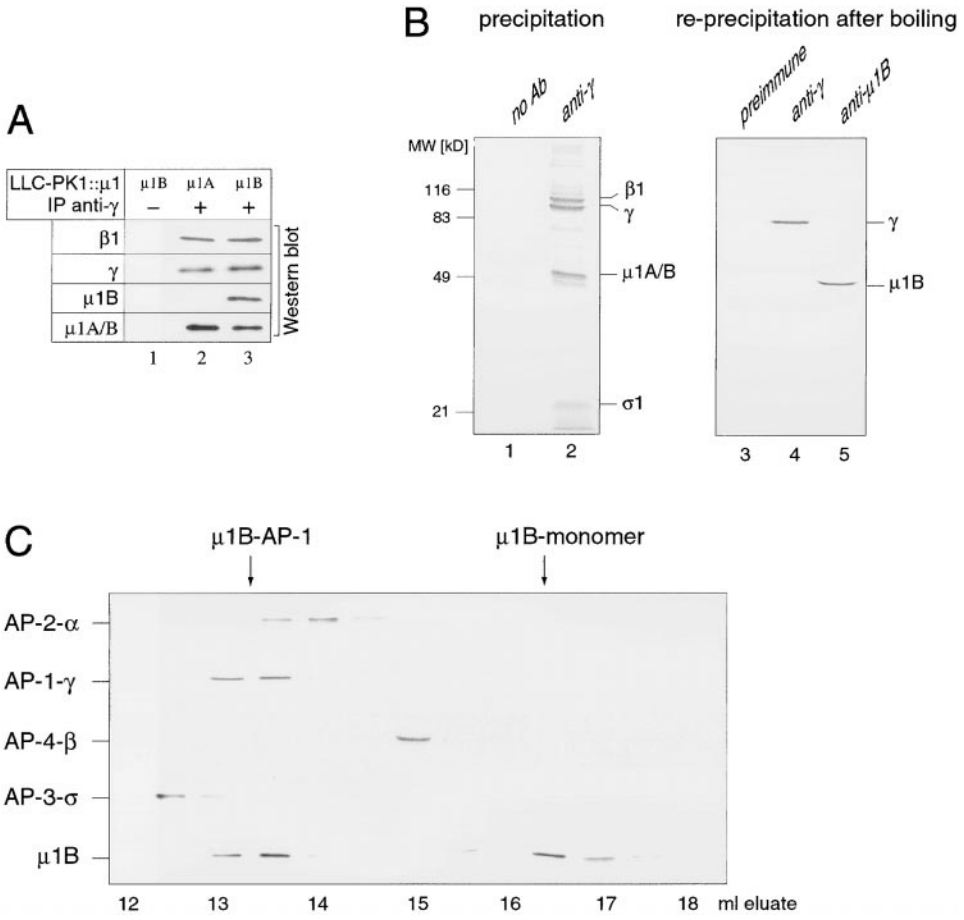

Figure 3. μ1B Is an Alternative Subunit of the Adaptor Complex AP-1

(A) LLC-PK1::μ1B transfectants (lanes 1 and 3) or LLC-PK1::μ1A transfectants (lane 2) were lysed in Triton X-100 buffer and subjected to coimmunoprecipitations using anti-γ-adaptin antibodies. Immunoprecipitates were analyzed by SDS–PAGE and immunoblotting using anti-β1/2-adaptin antibodies (clone 100/1), anti-γ-adaptin antibodies (clone 100/3, Sigma), μ1A/B cross-reacting antibodies, and μ1B-specific antibodies raised against the C terminus of μ1B.

(B) LLC-PK1::μ1B transfectants were metabolically labeled with [^{35}S]methionine/cysteine overnight and lysed in Triton X-100 buffer. AP-1 complex was precipitated using anti-γ-adaptin antibodies (clone 100/3). The precipitates were boiled in SDS buffer, and one-fourth was directly subjected to SDS–PAGE analysis. The remaining extract was diluted in lysis buffer, and subunits of the AP-1 complex were recaptured using preimmune antibodies, anti-γ-adaptin antibodies (clone 100/3), or anti-μ1B antibodies directed against the C terminus of μ1B. Immunoprecipitates were analyzed by SDS–PAGE and autoradiography.

(C) Gel filtration analysis of LLC-PK1::μ1B cell lysates. LLC-PK1::μ1B transfectants were lysed in Triton X-100 buffer and subjected to gel filtration analysis as described in Experimental Procedures. The proteins α-adaptin (subunit of AP-2), γ-adaptin (subunit of AP-1), β4 (subunit of AP-4), σ3 (subunit of AP-3), and μ1B were detected in the eluate fractions by Western blotting and immunolabeling using subunit-specific antibodies.

cells transfected with μ1A cDNA (LLC-PK1::μ1A) or with μ1B cDNA (LLC-PK1::μ1B) were lysed and analyzed by Western blotting. Both the parental and transfected cell lines were found to express equivalent amounts of γ-adaptin, one of the large subunits of AP-1 (Figure 2C). The cross-reacting anti-μ1A antibody revealed a slight increase of total μ1 levels in both LLC-PK1::μ1A (Figure 2C, lanes 4–6) and LLC-PK1::μ1B transfectants (Figure 2C, lanes 7–9) as compared to the nontransfected LLC-PK1 cells (Figure 2C, lanes 1–3). In both cases, the increases were 2- to 4-fold relative to endogenous μ1A.

Finally, the μ1B-specific antibody confirmed μ1B expression only in LLC-PK1::μ1B transfectants (Figure 2C, lanes 7–9, lower panel). Virtually no signal was detected in nontransfected LLC-PK1 or LLC-PK1::μ1A transfectants (Figure 2C, lanes 1–6). Although only one representative clone of LLC-PK1::μ1A and LLC-PK1::μ1B is shown, analysis of several independently isolated clones yielded essentially the same results.

μ1B Is an Alternative Subunit of the AP-1 Adaptor Complex

Since μ1A and μ1B are highly homologous (79% identity at the amino acid level) (Ohno et al., 1999), it seemed likely that μ1B might be a component of the ubiquitously expressed AP-1 complex. To determine whether this was the case, we immunoprecipitated AP-1 from LLC-PK1::μ1A transfectants or LLC-PK1::μ1B transfectants

using anti-γ-adaptin antibodies. As shown in Figure 3A, anti-γ-adaptin antibodies coprecipitated γ-adaptin, β1, and μ1A from lysates of LLC-PK1::μ1A cells (Figure 3A, lane 2). When cell lysate from LLC-PK1::μ1B transfectants was used, μ1B was also coimmunoprecipitated (Figure 3A, lane 3).

To further prove that μ1B is a subunit of an AP-1 complex, we next metabolically labeled LLC-PK1::μ1B cells with [^{35}S]methionine/cysteine. Cells were lysed, and immunoprecipitations using anti-γ-adaptin antibodies were performed. The primary coimmunoprecipitate was then boiled in SDS to dissociate the AP-1 complex. The boiled extract was diluted in lysis buffer and used for a second round of immunoprecipitations using either anti-γ or anti-μ1B antibodies.

As shown in Figure 3B, total immunoprecipitates contained labeled bands corresponding to each of the four expected AP-1 subunits: β1, γ, μ1A or μ1B, and σ1 (Figure 3B, lane 2). In the second precipitation of dissociated subunits, anti-γ-adaptin antibodies precipitated only γ-adaptin (Figure 3B, lane 4), while antibodies to the C-terminal μ1B peptide precipitated only μ1B (Figure 3B, lane 5); μ1B could also be recaptured using the antibody against the N terminus of μ1B (data not shown). These data strongly suggest that μ1B becomes incorporated into an AP-1 complex in the μ1B transfectants. When LLC-PK1::μ1A transfectants were used for this experiment, μ1B could not be precipitated in the second round (data not shown).

To exclude the possibility that μ1B also assembled into other known adaptor complexes (i.e., AP-2, AP-3, or AP-4), we performed gel filtration chromatography on cytosol from LLC-PK1::μ1B transfectants using a Superose 6 column. As indicated by Western blot analysis of column fractions, μ1B eluted in two peaks (Figure 3C). One peak coeluted with AP-1, as indicated by the position of γ-adaptin at an apparent molecular weight of 270 kDa. The second peak eluted with a molecular weight of ~65 kDa and thus presumably represented unassembled and possibly monomeric μ1B. Importantly, μ1B did not coelute with subunits indicative of any other adaptor complex, each of which exhibited different apparent molecular weights as reported previously (Dell'Angelica et al., 1997, 1999a). These data suggest that μ1B can only assemble into AP-1 complexes in the transfected LLC-PK1 cells, with overexpressed μ1B existing as unassembled monomer rather than entering AP-2, AP-3, or AP-4 complexes.

Thus, two alternative AP-1 complexes can exist: one containing the ubiquitously expressed μ1A (AP-1A), and the other the epithelial cell-specific μ1B (AP-1B). Unfortunately, our μ1B antibodies were not suitable for immunofluorescence microscopy, as found for all other anti-μ chain antibodies generated to date (Simpson et al., 1996; Dell'Angelica et al., 1999a, 1999b). It was, therefore, impossible to easily compare the intracellular distribution of AP-1A and AP-1B. We were able to demonstrate, however, that the two subunits were associated with markedly different targeting functions.

μ1B Confers Basolateral Polarity of LDL and Transferrin Receptors in LLC-PK1 Cells

Having demonstrated that μ1B expression in LLC-PK1 cells results in its incorporation in an AP-1 adaptor complex, we next asked whether μ1B expression would

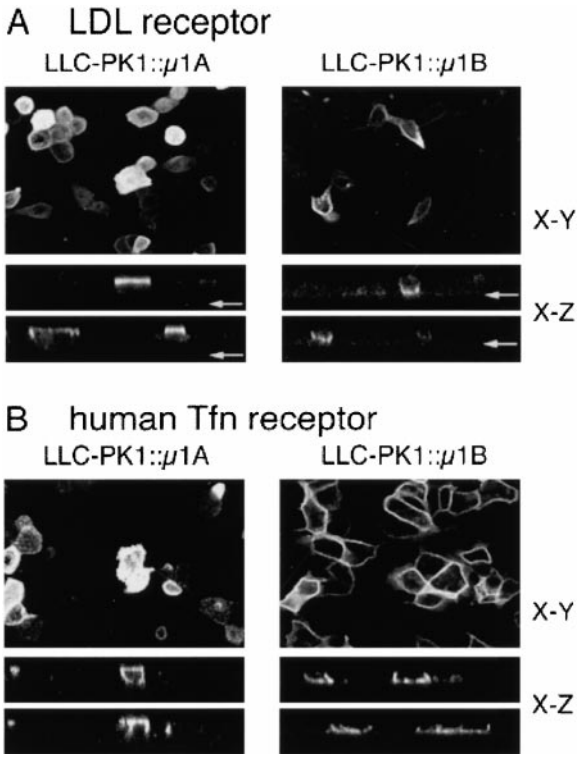

Figure 4. AP-1B Mediates Basolateral Sorting of LDL Receptor and Tfn Receptor

LLC-PK1::μ1A transfectants (left panels) or LLC-PK1::μ1B transfectants (right panels) were grown on Transwell filters for 4 days and infected with recombinant adenoviruses for 1 hr and cultured for 2 days to express LDL receptor (A) or human Tfn receptor (B). Cell surface staining with antibodies directed against LDL receptor or Tfn receptor, respectively, was achieved as described in Experimental Procedures. Specimens were analyzed by confocal microscopy, and representative X-Y and X-Z sections are shown. The arrows in (A) mark the position of the filters.

allow LLC-PK1 cells to localize LDL receptors at the basolateral surface. For this purpose, LLC-PK1 cells transfected with the μ1A or μ1B cDNAs were infected with adLDLR and analyzed for cell surface appearance of the receptor. As shown in Figure 4A (left panel), LLC-PK1 cells transfected with μ1A expressed the receptor only at the apical plasma membrane, as found for parental LLC-PK1 cells (Figure 1B). LLC-PK1 cells transfected with μ1B, however, exhibited a dramatic redistribution of LDL receptor to the basolateral plasma membrane (Figure 4B, right panel). Basolateral polarity was clearly evident in virtually all infected cells in both X-Y confocal images and X-Z vertical sections. These images were indistinguishable from those obtained for infected MDCK cells that express μ1B endogenously (Figure 1A).

Another well-studied basolateral receptor is the human transferrin receptor (Tfn receptor), which also relies on a cytoplasmic tail signal for targeting to the basolateral plasma membrane domain (Odorizzi and Trowbridge, 1997). We prepared a recombinant adenovirus encoding the wild-type receptor (adTfnR), infected μ1A- and μ1B-expressing LLC-PK1 cells, and then assayed for cell surface appearance of the receptor. As shown in Figure 4B (left panel), μ1A-expressing LLC-PK1 cells exhibited Tfn receptor at both the apical and basolateral

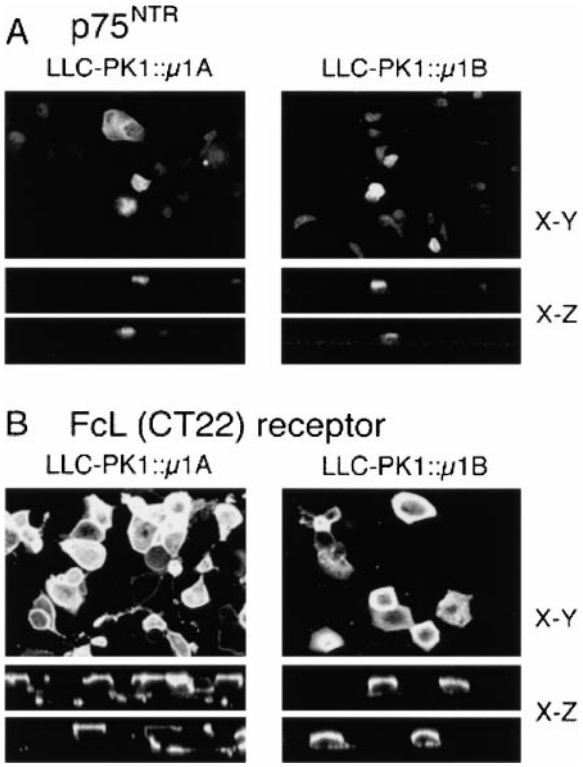

Figure 5. Apical Sorting Is Not Affected in LLC-PK1::μ1B Transfectants

LLC-PK1 cells transfected with μ1A cDNA (left panels) or μ1B cDNA (right panels) were grown on Transwell filters and infected with recombinant adenoviruses for 1 hr and cultured for 2 days to allow for expression of $p75^{NTR}$ (A) or FcL (CT22) receptor (B). Cell surfaces were stained with antibodies directed against the $p75^{NTR}$ or FcL receptor, respectively, and analyzed by confocal microscopy as described in Experimental Procedures. Shown are representative confocal images of X-Y and X-Z sections.

plasma membrane domains. This was consistent with the "random" phenotype of a tail-minus Tfn receptor mutant expressed in MDCK cells (Odorizzi and Trowbridge, 1997). In contrast, when adTfnR was used to infect μ1B-expressing LLC-PK1 cells, surface receptor was detected uniquely at the basolateral domain, a localization that was evident in every infected cell and clearly seen in both X-Y and X-Z confocal sections (Figure 4B, right panel). Thus, μ1B expression in LLC-PK1 cells confers the capacity for basolateral localization of another membrane protein that is well known to be sorted to the basolateral surface in MDCK cells.

Polarity of Apical Proteins Is Not Affected by μ1B Expression in LLC-PK1 Cells

To determine whether μ1B-induced basolateral sorting is selective for membrane proteins that bear basolateral targeting signals, we next infected the μ1A- and μ1B-transfected LLC-PK1 cells with viruses encoding proteins that are apical in MDCK cells. We first examined the polarized expression of the $p75^{NTR}$ isoform of nerve growth factor receptor. $p75^{NTR}$ contains an O-glycosylated stalk in its extracellular domain that is required for apical targeting (Yeaman et al., 1997). Transfected LLC-PK1 cells were infected with a recombinant adenovirus encoding $p75^{NTR}$ and assayed for surface expression of the receptor. As shown in Figure 5A, $p75^{NTR}$ was found exclusively at the apical surface of both μ1A- and μ1B-expressing LLC-PK1 cells.

We next examined the expression of a second protein that is strictly apical in MDCK cells. A recombinant adenovirus was constructed encoding a chimeric membrane protein consisting of the extracellular region and membrane anchor of the murine Fc receptor (FcRII) fused to the cytoplasmic domain of the LDL receptor, truncated at position 22 (FcL [CT22] receptor). This truncation has previously been shown to delete all basolateral targeting information without affecting the receptor's endocytosis signal (dependent on the tyrosine at position 18) (Matter et al., 1994). As found for $p75^{NTR}$, FcL (CT22) receptor was expressed largely at the apical surface of LLC-PK1 cells regardless of whether they had been transfected with μ1A or μ1B (Figure 5B).

Taken together, these results demonstrate that μ1B expression in LLC-PK1 cells was only able to induce the basolateral targeting of proteins that contained basolateral targeting signals. Thus, a μ1B-containing AP-1 adaptor complex appears to be required and perhaps responsible for signal-mediated basolateral sorting.

μ1B Expression Enhances Overall Organization of Polarized LLC-PK1 Cells

Not all membrane proteins depend on cytoplasmic domain targeting signals for sorting to the basolateral surface of epithelial cells. Some proteins, such as E-cadherin or the Na,K-ATPase, achieve basolateral polarity by homotypic interactions with proteins on adjacent cells or by interactions with the cytoskeleton at lateral surfaces subsequent to cell–cell contact (Mays et al., 1995). Although parental LLC-PK1 cells were unable to decode at least some cytoplasmic tail basolateral targeting signals, it was possible that they nevertheless formed a basolateral domain by other means. We therefore next determined the polarity of endogenous Na,K-ATPase in cells expressing μ1A or μ1B. In both cases, Na,K-ATPase distribution was largely restricted to the lateral plasma membrane, corresponding to sites of cell–cell contact (Figure 6A). Thus, a protein whose polarity apparently depends on its ability to interact with cytoskeletal elements appeared to reach the basolateral domain even in the absence of μ1B.

Closer inspection of LLC-PK1 cell monolayers stained with Na,K-ATPase antibody suggested that μ1B expression had the effect of yielding cells with a more regularly organized monolayer. To examine this more directly, μ1A- and μ1B-expressing LLC-PK1 cells were fixed and stained with FITC-coupled phalloidin, which outlines the overall shape of each cell by revealing the localization of filamentous actin. As shown in Figure 6B (left panel), μ1A-expressing cells (and their nontransfected parents) often grew on polycarbonate filters as irregularly shaped discontinuous cell sheets. Frequently, the cells piled up on each other several deep in a disorganized fashion. μ1B-expressing LLC-PK1 cells, on the other hand, grew in a far more regular fashion (Figure 6B, right panel). Although individual cells were not of uniform size (as is characteristic of MDCK cell cultures), they exhibited a strict monolayer arrangement and covered the filter surface in an essentially continuous fashion.

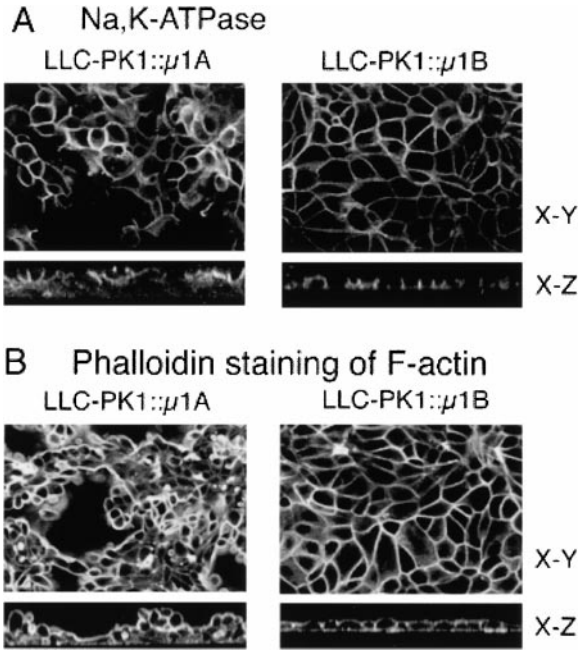

Figure 6. Expression of μ1B Induces More Regular Monolayer Growth

LLC-PK1::μ1A transfectants (left panels) or LLC-PK1::μ1B transfectants (right panels) were grown on Transwell filters for 4 days, fixed, and labeled with antibodies directed against the Na,K-ATPase (A) or with FITC-phalloidin (B). Specimens were analyzed by confocal microscopy, and representative X-Y and X-Z sections are shown.

These results suggest that LLC-PK1 cells are able to achieve some measure of apical and basolateral polarity even in the absence of μ1B. However, they are unable to correctly localize proteins containing basolateral targeting signals and also are unable to grow in regular monolayers unless μ1B was expressed by transfection.

Discussion

We have identified an important element of the mechanism responsible for targeting membrane proteins to the basolateral surface of epithelial cells. Our results suggest that final localization of a wide variety of membrane proteins at the basolateral surface depends on the expression of μ1B, an epithelial cell-specific component of the AP-1 clathrin adaptor complex. We propose that this AP-1B adaptor complex assembles with membranes at the level of the TGN, perhaps together with clathrin, to form nascent secretory vesicles that selectively accumulate proteins destined for the basolateral surface. The selective accumulation of cargo into these vesicles is likely to reflect the interaction of the AP-1B complex with at least a subset of distinct if degenerate targeting signals found in the cytoplasmic domains of many basolateral proteins.

Due to the leakiness of the monolayers of parental as well as transfected LLC-PK1 cells, it was impossible to perform the type of vectorial biotinylation experiments needed to establish directly that expression of AP-1B adaptors conferred the capacity to sort apical from basolateral proteins prior to their delivery to the plasma membrane. Nevertheless, it is highly likely that AP-1B adaptors do act at the level of sorting as opposed, for example, to selective retention of proteins following their appearance at the basolateral surface. First, the closely related AP-1A, AP-2, and AP-3 adaptor complexes are well known to mediate membrane protein sorting rather than retention (Mellman, 1996; Marks et al., 1997; Hirst and Robinson, 1998). In addition, the signals apparently utilized by AP-1B for basolateral targeting clearly act by facilitating polarized sorting, both in the TGN and in endosomes (Matter et al., 1993; Aroeti and Mostov, 1994). Indeed, the fact that the same signals are decoded at both sites suggests that the AP-1B adaptor complex similarly works in both the secretory and endocytic pathways. In this regard, it may be of interest that γ-adaptin has been found on clathrin-coated buds on endosomes as well as the TGN but not at the plasma membrane (Futter et al., 1998), although these earlier experiments could not distinguish AP-1A and AP-1B complexes.

AP-1B Complexes and the Decoding of Basolateral Targeting Signals

It seems likely that μ1B acts by directly recognizing basolateral targeting signals. Although this point remains to be directly demonstrated, it is by far the simplest explanation and consistent with the ability of μ chains to bind tyrosine-based signals for endocytosis and lysosomal targeting (Bonifacino and Dell'Angelica, 1999). Indeed, we first became interested in μ1B on the basis of a yeast two-hybrid screen in which the LDL receptor tail was challenged for interaction against all known μ chains. Only μ1B was found to interact, albeit very weakly, with the LDL receptor tail; the interaction was dependent on a tyrosine residue at cytoplasmic tail position 35 (R. C. Aguilar and J. S. B., unpublished data). In addition, preliminary chemical cross-linking experiments also suggest that the LDL receptor interacts with AP-1B, but not AP-1A, complexes in transfected LLC-PK1 cells (H. F. and I. M., unpublished data).

Regardless of whether μ1B functions by directly interacting with basolateral targeting signals, it is remarkable that μ1B can restore the polarized expression of such a wide array of basolateral proteins. While the LDL receptor clearly relies on critical tyrosine residues for basolateral sorting (Matter et al., 1992), Tfn receptor may not (Odorizzi and Trowbridge, 1997). Moreover, the fact that μ1B expression greatly improves the overall monolayer organization of LLC-PK1 cells suggests that it also induces the basolateral localization of one or more endogenous proteins (e.g., integrins) which play an important role in epithelial cell morphogenesis. In addition to such itinerant cargo proteins, μ1B may also be required for packaging of specific v-SNAREs required for efficient basolateral fusion.

In a yeast two-hybrid assay against peptides isolated from a combinatorial library, μ1B has been found to interact with a subset of tyrosine-containing motifs conforming to the canonical sequence YXXϕ (Ohno et al., 1999). This finding is consistent with the conservation of a YXXϕ-binding site on μ1B, as predicted from the crystal structure of μ2 (Owen and Evans, 1998). However, while some basolateral targeting signals, particularly those that are colinear with endocytosis signals,

appear to conform to this arrangement (Mellman, 1996), this seems not to be the case for the LDL or Tfn receptor basolateral signals. The LDL receptor tail contains two tyrosine-based, non-YXXϕ basolateral targeting determinants within its cytoplasmic tail (Matter et al., 1992). The membrane-proximal determinant comprises the endocytic signal NPVY and the acidic cluster EDE, while the membrane-distal determinant contains a GYSY sequence and an EDD acidic cluster. The Tfn receptor does contain a YXXϕ-type signal, YTRF, but this seems to mediate only endocytosis and not basolateral targeting (Odorizzi and Trowbridge, 1997). Targeting of the Tfn receptor to the basolateral surface is instead dependent, at least in part, on a GDNS sequence in the cytoplasmic tail (Odorizzi and Trowbridge, 1997). Thus, it is likely that the mode of binding of these basolateral targeting determinants to μ1B is distinct from that for YXXϕ signals. The failure of μ1A to substitute for μ1B in basolateral targeting could be explained by the absence of such a binding site for non-YXXϕ-type signals on μ1A.

Formation and Fate of AP-1B-Coated Vesicles

Given the close relationship between μ1A and μ1B, it seems likely that transport vesicles formed with AP-1B adaptors should contain clathrin, as is the case for "conventional" AP-1A-coated vesicles involved in TGN to endosome transport (Mellman, 1996; Hirst and Robinson, 1998). Basolateral clathrin-coated vesicles might thus contain both AP-1A and AP-1B adaptor complexes, suggesting their cargo might be similarly mixed. Indeed, in MDCK cells, lysosomal enzymes and membrane proteins when missorted to the plasma membrane are invariably targeted basolaterally, conceivably reflecting the partial inclusion of AP-1A-bound cargo in basolaterally targeted vesicles (Hunziker et al., 1991; Nabi et al., 1991). It remains possible that AP-1A and AP-1B complexes differ in other ways that ensure a better subdivision of their activities. For example, although the γ, β, and σ subunits that coprecipitate with μ1B are similar to the corresponding subunits in μ1A-containing complexes, they may also represent as yet unidentified, AP-1B-specific isoforms.

The existence of μ1B in epithelial cells strongly suggests that there are, in fact, some significant differences between how polarized and "nonpolarized" cells sort membrane proteins in the TGN. In epithelial cells, recognition of basolateral targeting signals by AP-1B appears crucial for the formation of transport vesicles competent for fusion with the basolateral plasma membrane. A protein not actively selected into these vesicles either because of lacking a sorting signal encoded in its cytoplasmic tail or because the respective adaptor complex is not expressed in the given cell line often segregates into glycolipid rafts and is subsequently delivered to the apical membrane. Studies in fibroblasts have suggested that the raft mechanism exists even in nonpolarized cells, leading to the segregation of cognate apical and basolateral proteins into distinct transport vesicles (Müsch et al., 1996; Yoshimori et al., 1996). However, the absence of μ1B expression in such cells would imply that the putative basolateral vesicles may, instead, actually be nonselective "default" vesicles, depleted of cognate apical markers. This could explain why export of basolateral proteins, such as VSV G protein, from the TGN of fibroblasts may not involve clathrin (Griffiths et al., 1985), although a role for clathrin in TGN to plasma membrane transport has not yet been definitively investigated in any cell type. The coats on such vesicles may be extremely evanescent, making their identification difficult by conventional techniques.

Polarized Sorting in the Absence of μ1B

Our results provide a plausible explanation for the recently described sorting "defect" in LLC-PK1 cells for two proteins bearing tyrosine-based sorting signals, the H,K-ATPase β subunit and an influenza hemagglutinin mutant. Although both proteins are sorted to the basolateral plasma membrane in MDCK cells, they are apical in LLC-PK1 cells (Roush et al., 1998). Thus, the basolateral transport of these proteins would appear dependent on μ1B.

Interestingly, AP-1B is apparently not required for the basolateral expression of another membrane protein, an IgG Fc receptor FcRII-B2, which contains a dileucine-type targeting signal (Hunziker and Fumey, 1994; Matter et al., 1994; Roush et al., 1998). At least in vitro, dileucine signals interact with β rather than μ subunits (Rapoport et al., 1998). This observation suggests that, under some circumstances, AP-1A adaptors may be able to mediate basolateral targeting, or that there are yet additional functional pathways remaining to be discovered.

That other, AP-1B-independent mechanisms for polarized targeting exist is without question. Both rat hepatocytes and hippocampal neurons are clearly polarized, with both relying on basolateral-type targeting signals for transport to their sinusoidal and somatodendritic surfaces, respectively (Weisz et al., 1992; Yokode et al., 1992; Jareb and Banker, 1998; Winckler and Mellman, 1999). Yet, neither cell type expresses μ1B mRNA (Ohno et al., 1999). It is possible that other cell type–specific μ or other adaptor subunits exist that mediate polarized sorting in hepatocytes and neurons. Another possibility relates to the existence of different strategies for polarized targeting. In hepatocytes, for example, basolateral and apical proteins are not sorted in the TGN but rather are delivered together to the sinusoidal (basolateral) plasma membrane and then sorted in endosomes following internalization (Hubbard et al., 1989; Wilton and Matthews, 1996). Perhaps the ability to interact with AP-1A, or an entirely novel sorting complex, at the level of endosomes precludes the need for AP-1B, which may be required for sorting only in the TGN. Thus, despite the clear importance of AP-1B in the organization of many types of epithelial cells, its role is played in the context of other mechanisms that act in concert to ensure the generation and maintenance of cellular asymmetry.

Experimental Procedures

Cloning and Expressing of μ1B and μ1A

All constructs were cloned by PCR using mouse μ1A cDNA (GenBank No. M62419) or human μ1B cDNA (I.M.A.G.E. consortium, LLDL, ID 123283) as templates and the Pfu-polymerase (Stratagene). Both genes were cloned into the bacterial expression vector pET-15b (Novagen) providing an N-terminal His_{6tag} for overexpression in

E. coli or into the CMV-based mammalian expression vector pCB6 for transfection of LLC-PK1 cells.

The N-terminal and C-terminal primers for cloning of μ1A into pET-15b were 5′-GCGCGAATTCCTCGAGATGTCCGCCAGCGCCGT CTACGTA-3′ and 5′-GCGCGGATCCTCACTGGGTCCGGAGCTG-3′, respectively. The PCR product was cloned as a XhoI/BamHI fragment. For cloning into pCB6, μ1A was amplified using the N-terminal primer as above and the following C-terminal primer 5′-GCGCAAG CTTTCACTGGGTCCGGAGCTG-3′ and cloned as an EcoRI/HindII fragment.

μ1B amplification for cloning into pET-15b was achieved by using the following N-terminal and C-terminal primers 5′-GCGCGAATTC CTCGAGATGTCCGCCTCGGCTGTCTTCATT-3′ and 5′-GCGCAGA TCTGTCGACCTAGCTGGTACGAAGTTGGTAATCGCC-3′, respectively, and cloned as a XhoI/BglII fragment. Cloning of μ1B into pCB6 was achieved using the same N-terminal primer as for cloning into pET-15b and 5′-GCGCAAGCTTCTAGCTGGTACGAAGTTG-3′ as C-terminal primer. The PCR product was cloned as an EcoRI/HindII fragment.

For overexpression in *E. coli*, the genes cloned into pET-15b were transformed into the *E. coli* strain BL21 (Novagen), overexpressed and partially purified under denaturing conditions using Ni-NTA chromatography.

For expression in LLC-PK1 cells, pCB6 harboring μ1A or μ1B cDNA was transfected using the calcium phosphate precipitation method as described (Hunziker et al., 1991). Positive transfectants were selected and maintained in growth medium supplemented with 1.8 mg/ml Geneticin.

Antibodies

μ1B-specific polyclonal antibodies were generated by injecting the peptides KGALAPLLSHGQVH and KEKEEVEGRPPIGV corresponding to the N-terminal or C-terminal region of the human μ1B protein, respectively, into rabbits. Polyclonal antibodies against μ1A were a gift of Linton Traub (Washington University). Polyclonal anti-$p75^{NTR}$ (9992) antibodies were obtained from Moses Chao (Skirball Institute). σ3 and β4 antibodies were as previously described (Dell'Angelica et al., 1997, 1999a). Monoclonal antibodies against β1/2 (clone 100/1), α-adaptin (clone 100/2), and γ-adaptin (clone 100/3) were purchased from Sigma.

Hybridoma cell lines producing the following monoclonal antibodies were purchased from the ATCC: anti-LDL receptor antibody (C7), anti-FcL receptor antibody (2.4G2), and anti-human Tfn receptor antibody (H68.4). Anti-Na,K-ATPase antibody (6H) was obtained from Michael Caplan (Yale University).

FITC-phalloidin and FITC-labeled secondary antibodies were purchased from Sigma or Jackson Immuno Research, respectively.

Cell Culture

LLC-PK1 cells were grown in α-MEM containing 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine at 37°C in a 5% CO_2 incubator. Stably transfected cell lines were maintained with 1.8 mg/ml Geneticin in the medium. For polarity experiments, cells were plated at a density of 8×10^5 cells per 24 mm filter on polycarbonate membrane filters (Corning-Costar Transwell units, 0.4 μm pore size) and cultured for 4 days with changes of medium every day.

The construction of adenoviruses encoding for human Tfn receptor or FcL (CT22) receptor (Matter et al., 1994) was done as described (He et al., 1998). The $adp75^{NTR}$ and adLDL receptor were gifts from Moses Chao (Skirball Institute) and James Wilson (University of Pennsylvania), respectively.

For infection with defective adenoviruses, cultures were washed once in serum-free medium, and 50–100 plaque-forming units (pfu) of the viruses was added to the apical chamber. After 1 hr incubation at 37°C, the medium was exchanged with serum-containing medium. The cells were prepared for immunofluorescence analysis 2 days after the infection.

For cell surface staining, the cultures were washed twice with PBS^{++} (PBS [2 g/l KCl, 2 g/l KH_2PO_4, 8 g/l NaCl, 1.15 g/l Na_2HPO_4, pH 7.4] plus 147 g/l $CaCl_2 \times 2\ H_2O$, 1 g/l $MgCl_2 \times 6\ H_2O$), and antibodies were added to the apical and basolateral side. After an incubation of 7.5 min at room temperature, cultures were washed twice with PBS^{++} and fixed in 3% paraformaldehyde/PBS^{++} for 15 min at room temperature. Subsequently filters were cut out and incubated in a blocking solution (2% BSA [w/v], 0.1% saponin [w/v] in PBS^{++}) for 1 hr followed by a 1 hr incubation with the fluorescently labeled secondary antibody diluted in blocking solution in a wet chamber. The filters were washed four times over a total period of 30 min in blocking solution and finally mounted in a glycerol solution (50% glycerol [w/v], 10% DABCO [w/v] in PBS). For total staining, the cultures were first incubated with the primary antibody for 1 hr, washed, and incubated with the secondary antibody as described above. The preparations were analyzed using a Zeiss confocal microscope (Microsystem LSM) with an Axiovert 100 microscope and a Zeiss Plan-Neofluar 40× 1.3 oil immersion objective.

Biochemical Procedures

For immunoprecipitations or gel filtration analysis, LLC-PK1 cells were split 1:1 or 1:2 in six-well plates 1 day prior to the experiment. The cells were washed twice with ice-cold PBS^{++} on ice. Buffer A (1% Triton X-100 [w/v], 0.3 M NaCl, 1× protease inhibitors [Boehringer], 50 mM Tris-HCl [pH 7.4], 0.1% BSA [w/v]) was added to the samples, the cells were scraped with a cell scraper, and then passed four times through a 22 gauge needle and a 1 ml syringe. Lysis was completed for 30 min on ice. A clarifying spin was done (15 min at 13,000 rpm, Eppendorf centrifuge at 4°C for immunoprecipitations or 30 min at 100,000 × g for gel filtration analysis), and the supernatant was used for further analysis. For Western blot analysis, cells were lysed in RIPA buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Triton X-100 [w/v], 0.5% desoxycholate [w/v], 0.1% SDS [w/v], 1× protease inhibitors [Boehringer]), and the protein concentration was determined.

For coimmunoprecipitations of μ1B with antibodies against γ-adaptin, the mouse monoclonal antibody 100/3 (Sigma) was bound to protein G–Sepharose. Lysis supernatant was added to the beads and incubated for 1 hr end-over-end at 4°C. Coimmunoprecipitates were washed two times with buffer A and once with buffer A without Triton X-100. The samples were analyzed by SDS–PAGE and immunoblotting.

For recapturing experiments, cells were labeled with 2 mCi/ml [^{35}S]methionine/cysteine (Promix, Amersham) in a mixture of 91% RPMI medium without methionine/cysteine (+10% [v/v] dialyzed FBS) and 9% αMEM (+10% FBS [v/v], +2 mM L-glutamine) for 14–16 hr prior to lysis. Immunoprecipitates obtained with anti-γ-adaptin antibody were washed five times in buffer A, and SDS buffer was added (0.1 M Tris-HCl [pH 7.4], 1% SDS [w/v], 10 mM DTT). The samples were vigorously shaken for 20 min at 4°C and boiled for 5 min at 95°C to release the immunoprecipitates from the antibodies and to denature the AP-1 complex. One-fourth of the extract was directly analyzed by SDS–PAGE. The remaining extract was diluted 20-fold with buffer A followed by a clarifying spin (15,000 rpm, 4°C, Eppendorf centrifuge) and incubated end-over-end at 4°C for 1 hr with anti-μ1B antibodies, the corresponding preimmune antibodies, or anti-γ-adaptin IgGs coupled to protein A or protein G–Sepharose, respectively. Immunoprecipitates were washed twice with buffer A and once with buffer A without Triton X-100. The samples were analyzed by SDS–PAGE and autoradiography.

For gel filtration analysis, cells from one chamber of a six-well plate were lysed in 400 μl of buffer A. Two hundred microliters of lysis supernatant was subsequently applied to a Superose 6 gel filtration column (25 ml column volume, Pharmacia) equilibrated with buffer B (0.5 mM EDTA, 0.5 mM PMSF, 1% Triton X-100, 0.3 M NaCl, 50 mM Tris-HCl [pH 7.4]). Fractions (0.5 ml) were collected and precipitated by adding TCA to a final concentration of 10% (w/v) and analyzed by SDS–PAGE and Western blotting.

Miscellaneous

SDS–PAGE was performed according to the published method of Laemmli (1970). Protein determination and detection of proteins after blotting onto nitrocellulose were performed using the BCA protein determination assay or the supersignal detection system, respectively, according to the supplier's instructions (Pierce).

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